Healing of Burn Wounds in Transgenic Mice Overexpressing Transforming Growth Factor- β 1 in the Epidermis

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Transforming growth factor- β (TGF- β) isoforms are multifunctional cytokines that play an important role in wound healing. Transgenic mice overexpressing TGF- β in the skin under control of epidermal-specific promoters have provided models to study the effects of increased TGF- β on epidermal cell growth and cutaneous wound repair. To date, most of these studies used transgenic mice that overexpress active TGF- β in the skin by modulating the latency-associated-peptide to prevent its association with active TGF- β . The present study is the first to use transgenic mice that overexpress the natural form of latent TGF- β 1 in the epidermis, driven by the keratin 14 gene promoter to investigate the effects of locally elevated TGF- β 1 on the healing of partial-thickness burn wounds made on the back of the mice using a CO₂ laser. Using this model, we demonstrated activation of latent TGF- β after wounding and determined the phenotypes of burn wound healing. We found that introduction of the latent TGF- β 1 gene into keratinocytes markedly increases the release and activation of TGF- β after burn injury. Elevated local TGF- β significantly inhibited wound re-epithelialization in heterozygous (42% closed versus 92% in controls, $P < 0.05$) and homozygous (25% versus 92%, $P < 0.01$) animals at day 12 after wounding. Interestingly, expression of type I collagen mRNA and hydroxyproline significantly increased in the wounds of transgenic mice, probably as a result of a paracrine effect of the transgene. (*Am J Pathol* 2001, 159:2147–2157)

The transforming growth factor- β (TGF- β) family of growth factors are potent regulators of cell growth and differentiation and play an important role in wound healing.^{1,2} TGF- β s exist in a number of structurally related but functionally distinct 25-kd homodimeric isoforms. In mammals, three isoforms, TGF- β 1, 2, and 3, have been

identified. Each isoform is synthesized as a large latent precursor that is unable to trigger signaling via high-affinity TGF- β receptors and is therefore named latent TGF- β .^{3,4} Latent TGF- β consists of a disulfide-linked dimer of 25-kd mature TGF- β associated with a 75-kd latency-associated-peptide (LAP). Activation of latent TGF- β , a process involving release of mature TGF- β from the latent precursor, is required for TGF- β to elicit its biological effects on the cells.⁵

TGF- β 1 is the predominant isoform in most tissues and is particularly abundant in platelets.³ After injury, high levels of TGF- β 1 are released from degranulating platelets. However sustained levels of TGF- β in wound tissue are subsequently produced by a number of other cell types present in wound, including macrophages, keratinocytes, fibroblasts, and endothelial cells.^{1–3} TGF- β acts via autocrine and paracrine mechanisms to regulate the interactions between cells and between cells and matrix in wound healing, involving inflammation, re-epithelialization, angiogenesis, and the production of extracellular matrix.^{1,2} Application of exogenous TGF- β either locally or systemically has been found to accelerate healing, particularly in chronic or impaired wounds.^{6,7} However, overexpression of TGF- β 1 has been implicated in various forms of fibrosis such as glomerulonephritis, liver cirrhosis, pulmonary cirrhosis,^{8–10} as well as hypertrophic scar.^{11–13}

To explore the effects of increased local TGF- β 1 on skin development and wound repair, various transgenic mouse models have been established using different keratin promoters to induce the overexpression of TGF- β 1 in the epidermis. To date, most investigations have been performed in transgenic mice that express the constitutively active TGF- β 1 by mutation of Cys-223→Ser and Cys-225→Ser in the LAP, thus preventing its binding to mature TGF- β .^{14–17} Constitutive overexpression of active TGF- β 1 in the epidermis, driven by the human keratin 1 promoter, results in neonatal lethality because of developmental deficiency in skin.¹⁸ Overexpression of

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TGF- β 1 in the epidermis driven by the keratin 6 or keratin 10 promoters gave contradictory results, either inhibiting or stimulating keratinocyte proliferation.^{14,16} Recently, Wang and colleagues¹⁷ reported a gene-switch system, in which the expression of the TGF- β 1 transgene in the epidermis was controlled by topical application of an inducer. This study suggests that induction of the TGF- β 1 transgene produces an inhibitory effect on keratinocyte growth in both hyperproliferative and quiescent cells. All of these studies focused on the effect of a persistent increase in active TGF- β on epidermal cell proliferation in nonwounded skin. However, phenotypes observed in these models may not necessarily reflect the physiological role of the latent form of TGF- β in wound healing.

In this study, we investigated the effects of locally elevated TGF- β 1 in laser-induced burn wound healing, using an established transgenic mouse model that overexpresses human latent TGF- β 1 in the epidermis, driven by a keratin 14 (K14) gene promoter. We also measured the amount of total and active TGF- β in the wound tissue by the plasminogen activator inhibitor/luciferase (PAI/L) assay and the distribution of TGF- β in the wounds by immunohistochemistry. Our data suggest that injury to the skin increases the release of TGF- β 1 from epidermal keratinocytes and activation of latent TGF- β . Overexpressed TGF- β 1 in the epidermis, through an autocrine pathway, inhibits keratinocyte proliferation, resulting in a marked delay in wound re-epithelialization. Furthermore, overexpression of TGF- β 1 in the epidermis increases the expression of type I collagen mRNA and hydroxyproline in the wound through a paracrine pathway.

Materials and Methods

Wound-Healing Model

Transgenic mice that overexpress a latent human TGF- β 1 gene in the epidermis driven by a K14 gene promoter were used in this study.¹⁹ The generation and characterization of this transgenic mouse strain has been described elsewhere (T. Chan and colleagues, manuscript submitted). Both heterozygous (HT) and homozygous (HM) animals were studied. Wild-type (WT) mice of the same strain were used as controls. Under metafer anesthesia, the dorsal surface of mouse was clipped, chemically depilated, and wiped with betadine and saline. Four histologically proven partial-thickness wounds (4 $\times$ 6 mm) were made in the dorsal skin using a gas-charged CO₂ laser set at 10 W, and 0.2-second exposure time. The animals were allowed to recover, housed separately, and fed *ad libitum* until the wounds were harvested. Wounds were examined visually and the time of closure was recorded. At days 0 (within 1 hour after wounding), 6, 12, 16, and 32 after wounding, six animals from each group were sacrificed by CO₂ overdose and the wounds harvested using a 6-mm punch biopsy. From each animal, one wound was fixed in 4% paraformaldehyde and prepared for histology and immunocytochemistry; two wounds were collected in 4 mol/L guanidinium isothiocyanate for RNA extraction; and one wound was

frozen for hydroxyproline analysis. For measurement of TGF- β in wound tissue by the PAI/L assay, three more animals were sacrificed at each time point and wound samples were processed as described below. All animal studies were conducted in compliance with Canadian Council on Animal Care guidelines and the University of Alberta Health Sciences Animal Policy and Welfare Committee regulations.

Histology and Immunocytochemistry

Hematoxylin and eosin (H&E) staining was performed on 6- μ m paraffin sections to confirm the partial-thickness wounds created by a CO₂ laser and assess the phenotype of wound healing by light microscopy. From each sample at least three sections through the center of the wound were examined for re-epithelialization.

The distribution of latent and active TGF- β in the skin and wounds was determined by immunohistochemistry in 6- μ m paraffin sections using the avidin-biotin immunoperoxidase staining technique. Expression of latent TGF- β 1 was examined using an antibody against human LAP1 (catalog no. AF-246-NA; R&D Systems, Minneapolis, MN). Active TGF- β 1 in the wounds was detected using an antibody against active TGF- β 1 (catalog no. AF-101-NA; R&D Systems). Sections were deparaffinized in xylene and rehydrated in graded ethanol. Endogenous peroxidase activity was quenched with 3% hydrogen peroxide in methanol (v/v) for 6 minutes at room temperature. Nonspecific protein binding was blocked with 10% normal serum from the same species as the biotinylated secondary antibody for 30 minutes. The primary antibody was applied to the slides at a final concentration of 20 μ g/ml in phosphate-buffered saline (PBS) and incubated overnight in a humidified chamber at 4°C. Control sections for LAP1 were incubated with nonimmune goat IgG. The specificity of active TGF- β staining was verified by incubation of the antibody with recombinant human TGF- β 1 (catalog no. 240-B; R&D Systems) before staining. After washing in PBS, sections were incubated with a biotinylated secondary antibody (1:150 dilution; Vector Laboratories, Burlingame, CA) for 40 minutes, followed by incubation with avidin-biotin complex (ABC kit; DAKO Diagnostics Canada Inc., Mississauga, Ontario, Canada) for 1 hour at room temperature. Sections were washed in PBS, then incubated for 3 minutes with 3-amino-9-ethylcarbazole for LAP1 or 0.05% (w/v) 3,3'-diaminobenzidine for active TGF- β . Sections were counterstained with hematoxylin and mounted with Permount for 3,3'-diaminobenzidine staining or AquaPerm mounting medium (Shandon, Pittsburgh, PA) for 3-amino-9-ethylcarbazole staining. Sections were viewed using a Nikon microscope and photographed using Kodak Ektachrome 200 ASA color films (Eastman-Kodak, Rochester, NY).

Detection of Proliferating Keratinocytes *in Vivo*

The effect of transgene expression on keratinocyte proliferation *in vivo* was analyzed by 5-bromodeoxyuridine (BrdU) incorporation at day 6 after wounding as de-

scribed.¹⁴ Mice were injected intraperitoneally with BrdU (Sigma) solution (250 μ g/g body weight in 0.9% NaCl) and killed 1 hour after injection. Wound specimens were frozen in Optimal Cutting Temperature compound. Cryosections (6 μ m) were immunostained with a mouse anti-BrdU antibody (RPN202; Amersham Pharmacia Biotech, Inc., Baie d'Urté, Quebec, Canada) followed by a biotinylated goat anti-mouse secondary antibody. The immunoreaction was visualized by ABC kit and 3,3'-diaminobenzidine. The number of BrdU-labeled keratinocytes per high-power field of the epidermis from the wound edge was counted.

Determination of Active/Total TGF- β Using the PAI/L Assay

To evaluate TGF- β activation during wound healing, we measured the levels of active and total TGF- β in cryosections of wounds by the PAI/L assay. This assay is based on the ability of TGF- β to induce plasminogen activator inhibitor-1 (PAI-1) expression in mink lung epithelial cells (MLECs) transfected with the PAI-1/luciferase construct.²⁰ Transfected MLECs were a generous gift from Dr. Daniel B. Rifkin (New York University Medical Center, New York, NY). MLECs were cultured in Dulbecco's modified Eagle's medium (Life Technologies, Inc., Grand Island, NY) supplemented with 5% fetal bovine serum, antibiotic-antimycotic (100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml amphotericin B; Life Technologies, Inc.) and 250 μ g/ml of geneticin (Life Technologies, Inc.). The cells were incubated at 37°C in an atmosphere of 5% CO₂. Cells between passages 10 and 30 were used for the PAI/L assay.

Wound samples were prepared from normal and transgenic mice at days 6, 12, and 16 after wounding as described.²¹ Briefly, four wounds, 4 $\times$ 4 mm for each, from one animal were embedded in one block of 1% sterile methyl-cellulose (Sigma Chemical Co., St. Louis, MO) and snap-frozen in liquid nitrogen. Four skin samples from the back of nonwounded normal and transgenic mice were also excised and processed as described above. The embedded tissue samples were stored at -80°C until used for analysis. When the PAI/L assay was performed, thick (24 μ m) cryosections were cut and placed on sterilized 13-mm round coverslips and temporarily stored at -20°C until transferred onto MLECs. Transfected MLECs were plated into 24-well cell culture dishes (3 $\times$ 10⁵/ml, 500 μ l/well) in complete Dulbecco's modified Eagle's medium and incubated for 4 hours at 37°C. Then, serum-containing medium was replaced with 500 μ l of Dulbecco's modified Eagle's medium containing 0.1% pyrogen-poor bovine serum albumin (Pierce, Rockford, IL), penicillin (100 U/ml), streptomycin (100 μ g/ml), and amphotericin B (0.25 μ g/ml). The coverslips each carrying four tissue sections from one animal were placed on MLECs with the sections facing down and incubated overnight. MLECs were lysed and luciferase activity was determined using a liquid scintillation counter (LS 6000TA; Beckman Instruments Canada Inc., Fullerton, CA) equipped with a single pho-

ton monitor. TGF- β levels were calculated by reference to a standard curve prepared with recombinant human TGF- β 1.

Total TGF- β in wound sections was measured after acidification.^{22,23} Coverslips carrying cryosections were placed in 24-well plates and submerged in 500 μ l of Dulbecco's modified Eagle's medium containing 0.1% bovine serum albumin. The samples were acidified with 12.5 μ l of 3 N HCl for 15 minutes at room temperature and neutralized with 35 μ l of 1 mol/L HEPES/5 N NaOH (5:2, v/v). The coverslips were transferred, together with the medium, onto MLECs for TGF- β quantification.

To normalize the amount of TGF- β to the wound size, each tissue specimen was trimmed to 4 $\times$ 4 mm and sectioned at 24 μ m in thickness. The wound area was measured under the microscope and no significant differences were observed between sections (data not shown).

Northern Analysis for Type I Procollagen mRNA

Total RNA was extracted from punch biopsies of wounds at selected time points after wounding. Tissue samples were lysed in 1 ml of 4 mol/L guanidinium isothiocyanate as previously described.¹³ Extracted RNA was then ethanol precipitated and used for Northern analysis. Ten μ g of total RNA extracted from wound tissue was loaded onto a 1% agarose gel and separated by electrophoresis. The RNA was transferred to nitrocellulose filters and baked at 80°C under vacuum for 2 hours. Filters were prehybridized in a solution containing 50% (v/v) formamide, 0.3 mol/L sodium chloride, 20 mmol/L Tris-HCl, pH 8.0, 1 mmol/L ethylenediaminetetraacetic acid, 1 $\times$ Denhardt's solution [1 $\times$ = 0.02% (w/v) bovine serum albumin, Ficoll, and polyvinylpyrrolidone], 0.05% (w/v) salmon sperm DNA, and 0.005% (w/v) poly(A) for 2 to 4 hours at 45°C. Hybridization was performed in the same solution for 16 to 20 hours at 45°C using a cDNA probe specific for the pro α 1(I) chain of type I procollagen (provided by Drs. G. Tromp, H. Kuivaniemi, and L. Ala-Kokko, Department of Biochemistry and Molecular Biology, Jefferson Institute of Molecular Medicine, Philadelphia, PA).¹³ The blots were subsequently rehybridized with cDNA specific for 18S ribosomal RNA (rRNA) as a control for loading. The probes were labeled with [α -³²P] dCTP (DuPont Canada, Mississauga, Ontario, Canada) by nick-translation. Filters were initially washed at room temperature with 2 $\times$ standard saline citrate (1 $\times$ = 0.15 mol/L sodium chloride, 0.015 mol/L sodium citrate) and 0.1% sodium dodecyl sulfate for 30 minutes and then for 20 minutes at 65°C in 0.2 $\times$ standard saline citrate and 0.1% sodium dodecyl sulfate. Autoradiography was performed by exposing Kodak X-Omat film to the nitrocellulose filters at -70°C in the presence of an enhancing screen. The cDNA probe for 18S ribosomal RNA was obtained from the American Type Culture Collection (Rockville, MD).

Figure 1. Immunolocalization of latent TGF- β 1 in normal skin and wounds. Cryosections of the skin and wounds taken from transgenic and WT mice were immunostained with an antibody specific for human LAP1. **A:** In normal skin of WT mice, faint staining of LAP1 was seen in the epidermis and mainly extracellular in distribution. In the skin of HT and HM mice, high intensity of LAP staining is located mainly in the basal keratinocytes (arrows) and has a predominantly intracellular distribution. **B:** In day 6 wounds, latent TGF- β was observed throughout the epidermis adjacent to wound in transgenic mice, whereas, it was located mainly in cytoplasm of suprabasal keratinocytes in WT controls. Original magnifications, $\times 400$.

Hydroxyproline Assay

The content of collagen in wound tissue was determined by mass spectrometric analysis for 4-hydroxyproline.²⁴ Wound samples taken from normal and transgenic mice at days 6, 12, and 16 after wounding were freeze-dried. Internal standard (*N*-methyl-L-proline) and 6 N HCl solution was added to wound tissue, and each sample was then hydrolyzed overnight at 115°C. The *O*-butyl ester derivatives were prepared with 10% BF₃-butanol for 30 minutes at 120°C after drying the hydrolysate. Liquid chromatography (column: Eclipse XDB-C18)/mass spec-

trometry analysis was performed on a Hewlett-Packard (series 1100, Atlanta, GA) mass selective detector monitoring the ions *m/z* 188.

Statistical Analysis

The data on wound re-epithelialization were analyzed using Fisher's exact test. The levels of active/total TGF- β , the number of BrdU-labeled keratinocytes and relative expression of type I collagen mRNA and hydroxyproline were compared using a one-way analysis

Figure 2. Immunolocalization of active TGF- β 1 in the wounds. Wound sections taken at day 6 after wounding were immunostained with an antibody against active TGF- β 1. In the wounds in WT mice (**A** and **B**), active TGF- β 1 was present mainly in the suprabasal keratinocytes of the migrating epithelial sheet. The intensity of TGF- β staining was increased in wounds made in HT (**C** and **D**) and HM (**E** and **F**) mice and distributed throughout the epidermis. Note the wide epithelial gap in the wounds in transgenic mice. Original magnifications: $\times 20$ (**A**, **C**, and **E**), $\times 100$ (**B**, **D**, and **F**).

of variance. For all comparisons $P < 0.05$ was considered to be significant. All data are expressed as mean values $\pm$ SEM.

Results

Immunolocalization of the K14-TGF- β Transgene in the Skin and Wounds

To verify expression of the transgene at the protein level, immunostaining for human latent TGF- β 1 was performed in nonwounded skin and wounds using an antibody against human LAP1. In nonwounded skin, a strong stain-

ing of latent TGF- β 1 was seen in basal keratinocytes of both HT and HM mice (Figure 1A). The staining pattern was mainly intracellular in distribution. In normal skin of WT mice, immunostaining of latent TGF- β was visible in the epidermis but the intensity of staining was markedly less than that in transgenic mouse skin (Figure 1A). At day 6 after skin injury, staining for latent TGF- β 1 was increased in the suprabasal keratinocytes in WT mice (Figure 1B). At this time point, latent TGF- β staining was especially strong and present throughout the epidermis adjacent to the wounds of transgenic mice (Figure 1B). Similar differences in the intensity and localization of latent TGF- β staining were also observed between wounds in transgenic and WT mice on day 12 (data not shown).

Immunostaining for Active TGF- β in the Wounds

Because activation of latent TGF- β is a key point in the regulation of TGF- β action, we evaluated active TGF- β in the skin and wounds by immunohistochemistry. In non-wounded skin, no active TGF- β was detectable (data not shown). After wounding, marked differences in the intensity and localization of TGF- β staining were observed between wounds in transgenic and WT mice. In day 6 wounds, active TGF- β was found in the migrating epithelial sheet in both WT and transgenic mice (Figure 2). In wounds in WT mice, active TGF- β was present mainly in the suprabasal keratinocytes, whereas, in wounds in transgenic mice, TGF- β was distributed throughout the epidermis. The same staining patterns were also observed in day 12 wounds (data not shown). The specificity of TGF- β staining was confirmed by the fact that 100 μ l of antibody (20 μ g/ml) was completely neutralized by incubation with 200 μ g of recombinant human TGF- β overnight at 4°C (data not shown).

The Levels of Total and Active TGF- β in the Wounds

To further quantitate the amount of total and active TGF- β in the wounds, we further analyzed samples taken from WT and transgenic mice before and after injury using the PAI/L assay. The levels of total TGF- β were significantly higher in nonwounded skin and wounds of transgenic mice compared with WT controls. In WT mice, the amount of total TGF- β was twofold to threefold higher in wounds than in normal skin at days 6, 12, and 16 after wounding, whereas this increase was significantly higher in wounds of HT and HM mice at these time points (Figure 3A). However, there were no significant differences in the amount of active TGF- β in nonwounded skin for transgenic and WT mice. After injury, the level of active TGF- β markedly increased in all wounds, however, the increase was more apparent in wounds of transgenic mice compared with controls. At day 6, there was a very significant increase in active TGF- β in HT (15.9 ± 1.1 pg/section, $P < 0.01$, $n = 12$) and HM (18.8 ± 2.9 pg/section, $P < 0.01$, $n = 12$) mice, compared to normal controls (4.5 ± 1.7 pg/section). Although the levels of active TGF- β in the wounds of transgenic mice remained higher than those in WT wounds at days 12 and 16, the differences were not statistically significant (Figure 3B).

Microscopic Features of the Laser Wounds

First, we determined the time course and morphological changes of partial-thickness burn wounds created by a CO₂ laser in WT mice by visual observation and light microscopy. Histologically, the epidermis on the wound surface and collagen in the papillary dermis were destroyed soon after wounding (within 1 hour) with a thick layer of fatty tissue remaining under the dermis. There were no immediately visible changes in the panniculus (Figure 4A). At day 6, the wounds remained open and

Figure 3. The levels of total and active TGF- β in the skin and wound sections. Cryosections of the skin and wounds taken at days 6, 12, and 16 after wounding from WT, HT, and HM mice were analyzed for total (A) and active (B) TGF- β using the PAI/L assay. The data represent mean values $\pm$ SEM of three separate experiments, each examined in triplicate with significant differences compared to controls at * $P < 0.05$ or ** $P < 0.01$ (analysis of variance).

were characterized by a large number of infiltrated inflammatory cells. The keratinocyte layers at the wound edges were becoming thicker and the panniculus was no longer present at the wound sites (Figure 4B). Re-epithelialization was complete in majority of the wounds at day 12, at which time high cellularity was still obvious and some new collagen was seen within the wounds (Figure 4C). By day 16, the wound areas became smaller, with dense granulation tissue present (Figure 4D). By day 32, cellularity was markedly reduced and scar tissue was seen at the wound site (data not shown).

Retarded Wound Re-Epithelialization in Transgenic Mice

Next, we examined the extent of wound closure and re-epithelialization in transgenic mice (48 wounds in each group). It was found that the extent of re-epithelialization was markedly inhibited in wounds in HT and HM mice compared with WT controls. At day 6, the epithelial gap between the leading edges of wound was wider in transgenic mice compared with that in WT mice (Figure 2). At day 12, the majority of the wounds in transgenic mice had re-epithelialized (Figure 5A). However, the re-epithelialization was markedly delayed in wounds in transgenic mice. As shown in Figure 5B, 92% of the wounds in WT

Figure 4. Histological features of laser wound healing in WT mice. Partial-thickness wounds were created on the back of the mice using a CO₂ laser. Mice were sacrificed at different time points after wounding. Paraffin sections of the wounds were stained with H&E. **A:** Immediately after wounding the epidermis and dermis were damaged with a thick layer of fatty tissue remaining underneath. No immediate injury to the panniculus was observed. **B:** Day 6 wounds were characterized by infiltration of large number of inflammatory cells (arrow). The keratinocyte layers became thicker at the wound edge (arrowhead). The panniculus was no longer present at the wound sites. **C:** At day 12, re-epithelialization was complete with thick neo-epidermis at the wound surface. **D:** By day 16 the wound area became smaller and dense granulation tissue was seen in the wound. e, epidermis; f, fatty tissue; p, panniculus; gt, granulation tissue. Original magnifications, $\times 20$.

mice had closed, whereas, only 42% of wounds in HT and 25% in HM mice were closed ($P < 0.05$) at day 12. The retarded wound re-epithelialization reflects the levels of expression of transgene in HT and HM animals at this time point. At day 16, all wounds of WT mice had re-epithelialized, at which time although the extent of wound re-epithelialization was lower (67%) in transgenic animals, this difference did not reach statistical significance (Figure 5B).

TGF- β Transgene Inhibits Keratinocyte Proliferation in Vivo

To determine the mechanisms underlying the effects of the transgene on wound re-epithelialization, we analyzed the proliferation of the keratinocytes *in vivo* by labeling with BrdU in day 6 wounds. As showed in Figure 6, BrdU-labeled cells were mainly restricted to the basal layer of the epidermis. The number of proliferating keratinocytes at the wound edge in transgenic mice was significantly lower than that in WT controls (11.6 ± 2.1 per high-power field in WT *versus* 7.1 ± 1.4 in HT, $P < 0.01$, $n = 9$; *versus* 5.9 ± 1.6 in HM mice $P < 0.01$, $n = 9$). These data indicate that the overexpression of TGF- β 1 in the skin inhibits keratinocyte proliferation *in vivo* during cutaneous wound healing.

Increased Expression of Type I Collagen mRNA in Transgenic Wounds

The relative expression of type I procollagen mRNA was compared by Northern analysis in wound tissue taken from transgenic and WT mice at days 16 and 32 after wounding. As shown in Figure 7A, there were two different transcripts, 5.8 kb and 4.8 kb, for type I procollagen mRNA (top row), consistent with our previous report.¹³ The level of type I procollagen mRNA was markedly increased in the wounds in HT and HM mice compared to WT controls. Rehybridization of the same blot with a cDNA specific for 18S rRNA (Figure 7A, bottom row) showed that loading of total RNA was similar for all samples examined. Autoradiograms were quantified by densitometry, and the relative intensity of type I collagen mRNA was corrected for variations in loading using the 18S rRNA band. Significant increases in expression of type I collagen mRNA were seen in the wounds in HT and HM mice, compared to WT controls at day 16 (densitometry units: 2.79 ± 0.27 in HT *versus* 2.04 ± 0.12 in WT, $P < 0.05$; 2.65 in HM *versus* 2.04 ± 0.12 in WT, $P < 0.05$, $n = 3$) and day 32 (3.78 ± 0.17 in HT *versus* 2.46 ± 0.17 in WT, $P < 0.01$; 3.88 ± 0.22 in HM *versus* 2.46 ± 0.17 in WT, $P < 0.01$, $n = 3$) after wounding (Figure 7B).

Figure 5. Retarded wound re-epithelialization in transgenic mice. **A:** Representative wound sections taken from WT, HT, and HM mice at day 12 after wounding illustrate delayed re-epithelialization in transgenic mice. **B:** Wound closure in WT, HT, and HM mice was assessed visually and histologically. Wounds were scored as open or closed. Forty-eight wounds were examined in each type of animal at 12 days and 16 days (Fisher's exact test; *, $P < 0.05$; **, $P < 0.01$).

Figure 6. Detection of proliferating cells in the epidermis of wounds by BrdU labeling. At day 6 after wounding, WT (**A**) and transgenic (**B**) mice were injected intraperitoneally with BrdU 1 hour before sacrifice. Wound sections were stained with an anti-BrdU antibody. BrdU-labeled nuclei are indicated by **arrows**. Original magnification, $\times 100$.

Hydroxyproline Content in the Wounds

To verify the production of collagen at the protein level, liquid chromatography/mass spectrometry was used to compare the amount of hydroxyproline in wound tissue from WT, HT, and HM mice. It was found that the amount of hydroxyproline was significantly higher in HM mouse wounds compared to normal controls (14.7 ± 1.9 versus 5.6 ± 1.5 $\mu\text{g}/\text{wound}$, $P < 0.05$, $n = 4$) at day 16 (Figure 8).

Discussion

Our transgenic mice that overexpress latent TGF- β 1 in the skin driven by a K14 promoter have provided a useful model to determine TGF- β activation and the effects of locally elevated TGF- β on cutaneous wound healing. Expression of K14-TGF- β transgene at the protein level was demonstrated by immunohistochemistry in nonwounded skin. The intensive staining of latent TGF- β 1 observed in the basal keratinocytes of transgenic mice is consistent with previous reports that the transgene driven by a K14 promoter was targeted to the nondifferentiated basal keratinocytes in the epidermis.^{25,26} Very faint staining of latent TGF- β 1 observed in WT epidermis

Figure 7. Expression of mRNA for type I collagen in the wounds. Total RNA was extracted from each wound taken from WT, HT, and HM mice at days 16 and 32, and type I procollagen mRNA was determined by Northern analysis. **A:** The autoradiogram shown is representative of three autoradiograms from separate experiments, demonstrating the pattern of 5.8-kb and 4.8-kb transcripts (**top row**) corresponding to pro α 1(I) procollagen mRNA. The **bottom row** shows the profile of the 18S ribosomal RNA used as a control of RNA loading, obtained by rehybridization of the blot shown in the **top row**. **B:** Quantitative comparison of procollagen mRNA expression in WT, HT, and HM wounds. The relative intensity of procollagen mRNA to 18S ribosomal RNA was calculated and shown as the mean $\pm$ SEM with significant differences at * $P < 0.05$ or ** $P < 0.01$ (analysis of variance).

may reflect the expression of endogenous TGF- β and some cross-species cross-reactivity of the antibody to human LAP1. After injury, immunostaining of latent TGF- β was markedly increased in the epidermis of both transgenic and WT mice, indicating that keratinocytes are the major cellular source of TGF- β in cutaneous wound healing.

Latent TGF- β must be activated before exerting its biological effects on target cells. To determine whether overexpression of the latent TGF- β 1 in the skin results in an increase in active TGF- β formation after wounding, we also performed immunostaining for active TGF- β 1 in wound specimens. The location of active TGF- β in the suprabasal keratinocytes observed in the wounds in WT mice is in agreement with previous report.²⁷ However, the strong TGF- β staining present in all epidermal keratinocytes adjacent to the wound seems unique for our transgenic mice. These observations indicate that overexpression of latent TGF- β in the skin may cause autoinduction and activation of this cytokine during wound healing.

Figure 8. The levels of hydroxyproline in wound tissue. Wound samples taken from WT, HT, and HM mice at different time points were processed for mass spectrometric analysis for 4-hydroxyproline. The data represent mean values $\pm$ SEM of four separate experiments with a significant difference at * $P < 0.05$ (analysis of variance).

Based on our immunostaining results, we further measured the amount of total and active TGF- β in wound sections using the PAI/L assay. Our results demonstrate that the levels of both total and active TGF- β were dramatically increased in the transgenic mice after injury. However, the time course of total and active TGF- β generation were not parallel. Total TGF- β was persistently higher in the wounds made in transgenic mice, whereas a significant increase in active TGF- β was observed only in day 6 wounds in transgenic animals and then gradually declined. This implies a complex process of TGF- β production, activation, and clearance during wound repair. Although latent TGF- β driven by K14 promoter was constitutively expressed in the skin and the expression was dramatically increased after injury, the activation of TGF- β is tightly regulated by posttranscriptional mechanisms. In addition, the active form of TGF- β may rapidly bind to TGF- β receptors and be internalized, or degraded by proteases, leading to rapid elimination from the wound.

Although inhibition of keratinocyte proliferation by TGF- β 1 has been clearly demonstrated *in vitro*,^{28,29} studies in transgenic mice overexpressing TGF- β 1 *in vivo* have shown inconsistent and contradictory results.¹⁴⁻¹⁷ It was reported that epidermal-specific induction of the TGF- β 1 transgene decreased the DNA labeling index in the epidermis by sixfold.¹⁷ In contrast, transgenic mice with keratin 10 promoter-driven TGF- β 1 overexpression showed a twofold to threefold increase in the epidermal DNA labeling index in the absence of hyperplasia, but showed a growth inhibitory response during induction of hyperplasia.¹⁴ Our data demonstrated that local overexpression of TGF- β 1 inhibited wound re-epithelialization. Labeling of the keratinocytes *in vivo* with BrdU indicated that proliferation was significantly inhibited in transgenic animals. These observations are consistent with a recent report indicating that disruption of TGF- β signaling by knocking out the Smad3 gene in mice increased the rate of re-epithelialization of incisional wounds.³⁰

The ability of TGF- β to stimulate production of a wide spectrum of matrix proteins have been well demonstrated in many experimental models.^{6,7} However, it remains an open question whether TGF- β overexpressed in the epidermis may pass through the basement membrane and induce a paracrine effect on dermal cells. In the present study, we found that expression of type I collagen mRNA and hydroxyproline content were significantly increased in the wounds of transgenic mice. These results indicate that TGF- β released from the keratinocytes after wounding may penetrate to the underlying dermis and stimulate fibroblasts to produce collagen. Our data are supported by a recent study showing that transgenic mice overexpressing activin A, a member of the TGF- β superfamily, in the epidermis display abnormalities in the dermis.³¹ This might be because of the diffusion of activin A from the keratinocytes into the underlying dermis, possibly facilitated by abnormalities in the basement membrane. Using a gene-switch system, Wang and colleagues also observed a paracrine stimulating effect of TGF- β transgene on angiogenesis, however, they did not observe any fibrosis.¹⁷ The possible reasons for the different results include the different experimental models and TGF- β 1 constructs used in these two studies. The TGF- β 1 transgene used by Wang and colleagues¹⁷ is a constitutively active mutant TGF- β 1, and the expression of the transgene is induced by topical application of an inducer. In the undamaged skin, TGF- β 1 produced by keratinocytes would have to cross the basement membrane to exert a paracrine effect on the fibroblasts. It is known that the half-life of mature TGF- β is only 2 to 3 minutes.³² Because of its short half-life, active TGF- β may be rapidly eliminated from the wound before reaching the dermis. We investigated the effects of overexpression of latent TGF- β 1 in a wound-healing model rather than in intact skin. The release of TGF- β from keratinocytes is induced by wounding. As the basement membrane was damaged, TGF- β produced by keratinocytes may readily diffuse to the underlying dermis before re-epithelialization is complete. The transgenic mice used in the present study overexpress latent TGF- β 1. Because association of mature TGF- β 1 with the LAP prolongs the half-life to 100 minutes,³³ it is likely that LAP may facilitate the infiltration of TGF- β 1 into the dermis through the basement membrane. Therefore, the amounts of latent TGF- β 1 expected to diffuse to the dermis from the epidermis were much higher in our model. At present, the mechanisms of latent TGF- β activation *in vivo* are not fully understood but there is some evidence for the involvement of plasmin.³⁴ Plasmin-mediated TGF- β activation occurs on the cell surface and requires binding of latent TGF- β to the cell surface mannose-6-phosphate/insulin-like growth factor II receptor via mannose-6-phosphate residues in LAP.³⁵ Recently, a study on thrombospondin-1 knockout mice suggests that this molecule might be a major activator of TGF- β *in vivo*.³⁶ The levels of both plasmin and thrombospondin-1 are up-regulated at the wound site.^{37,38} In addition, it was found that small latent TGF- β 1 (mature TGF- β 1 + LAP) may bind to the fibrin clot formed in the wound via interaction of the RGD sequence in the LAP with the platelet integrin, GPIIb/IIIa.³⁹ This bound

TGF- β 1 may be gradually activated and released when the fibrin clot is dissolved by plasmin, resulting in a sustained presence of TGF- β 1 in the wound. Overexpression of TGF- β 1 in the epidermis may increase the retention of latent TGF- β 1 in the fibrin clot that may act as a sustained release form of TGF- β 1 activity during wound healing. This might be another reason for our observation of an increase in collagen production in HM transgenic mice. However, further experiments will be necessary to examine the later effects of TGF- β on dermal collagen metabolism.

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